Myosin Cross-Bridge Kinetics and the Mechanism of Catch

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ABSTRACT Catch force in molluscan smooth muscle requires little, if any, energy input and is controlled by the phosphorylation state of the thick filament-associated mini-titin, twitchin. The kinetic parameters of myosin cross-bridge turnover in permeabilized catch muscle, and how they are potentially modified by the catch mechanism, were determined by single turnover measurements on myosin-bound ADP. Under isometric conditions, there are fast and slow components of cross-bridge turnover that probably result from kinetic separation of calcium-bound and calcium-free cross-bridge pools. The structure responsible for catch force maintenance at intermediate [Ca^{+2}] does not alter the processes responsible for the fast and slow components under isometric conditions. Also, there is no measurable turnover of myosin-bound ADP during relaxation of catch force by phosphorylation of twitchin at pCa $>$ 8. The only effects of the catch link on myosin-bound ADP turnover are 1), a small, very slow extra turnover when catch force is maintained at very low [Ca^{+2}] (pCa $>$ 8); and 2), attenuation of the shortening-induced increase in turnover at subsaturating [Ca^{+2}]. These limited interactions between the catch link and myosin cross-bridge turnover are consistent with the idea that catch force is maintained by a thick and thin filament linkage other than the myosin cross-bridge.

INTRODUCTION

The catch state in molluscan smooth muscle is characterized by force maintenance and high resistance to stretch associated with little, if any, energy usage. Catch force can be maintained when intracellular [Ca^{+2}] is very low (1), and it is relaxed by serotonergic agents (2) that cause an increase in cAMP (3). The target of the cAMP-activated protein kinase is the thick filament-associated mini-titin, twitchin, whose phosphorylation state controls catch (4,5). When twitchin is unphosphorylated, catch force is maintained; and when twitchin is phosphorylated, catch force is relaxed. Activation of myosin cross-bridge cycling in molluscan catch muscles results from calcium binding to myosin (for review, see (6,7)), and at every subsaturating [Ca^{+2}], there is a catch component of force output which is relaxed by phosphorylation of twitchin (5).

The mechanism of catch force maintenance is not known. In vitro assays show that synthetic thick filaments containing purified myosin and twitchin tightly bind F-actin when twitchin is unphosphorylated, but not when twitchin is phosphorylated (8,9). This suggests that the catch-force maintaining link between thick and thin filaments is twitchin, the myosin cross-bridge or a combination of the two. If the link is the myosin cross-bridge, it is clear that catch force maintenance does not require calcium-activated myosin cross-bridge cycling. But, the link could be a non- or very slowly cycling actin-attached calcium-free myosin cross-bridge whose interaction with actin may depend on the phosphorylation state of twitchin (10,11). In this scenario, the same structure responsible for active force development (the myosin cross-bridge) is responsible for linking the thick and thin filaments in catch (see (12) for an early review of this idea). This would require that twitchin control at least some kinetic parameters of the myosin cross-bridge. On the other hand, there is evidence against such a model (13–15) and it may be that the twitchin molecule itself is the catch link between thick and thin filaments. There is some evidence that twitchin interacts with the thin filament in a phosphorylation-dependent manner (16). In this model, myosin cross-bridges are responsible for force development, but a twitchin link between the filaments maintains force when the cross-bridges detach (see (17) for an early review of the possibility that a protein other than actin and myosin is responsible for catch force).

The experiments reported here were performed to measure myosin cross-bridge turnover in catch muscle under various mechanical conditions and to determine whether the catch mechanism affects the kinetic parameters of the myosin cross-bridge. The results specifically allow identification of any unique characteristics of myosin turnover in muscles demonstrating catch, and, importantly, allow determination of whether the specialized mechanism responsible for catch force maintenance interacts with and regulates myosin cross-bridge function. In these experiments, the turnover of myosin was measured as the single turnover of myosin-bound ADP. This method deals directly with myosin cross-bridge turnover and allows the identification and characterization of different pools of myosin cross-bridges with unique cycling rates. The method was previously used in permeabilized mammalian smooth muscle to study the mechanism of regulation of myosin cross-bridge cycling by myosin light chain phosphorylation (18,19). The results showed that under isometric conditions, there was a fast cycling group of

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cross-bridges whose number was directly proportional to the extent of myosin light chain phosphorylation (19). But, there was also a slow cycling group of cross-bridges with unphosphorylated myosin light chains whose cycling rate was related to the extent of myosin light chain phosphorylation of the entire cohort of myosin. The results led to the postulation that myosin with unphosphorylated light chains is cooperatively activated by myosin with phosphorylated light chains in the same thick filament (18).

The basic premise of the single turnover experiments as performed on permeabilized muscle is that the large majority of myosin has ADP bound at any given time, and that the time course of release of the bound ADP and replacement with new ADP subsequent to ATP binding and splitting gives an accurate measure of myosin kinetics. Experiments were performed under steady-state conditions (e.g., during isometric force maintenance) where there is no net change in the distribution of myosin in various cross-bridge states during the single turnover protocol. In this case, measurement of the single turnover of myosin-bound ADP allows determination of 1), the rate at which cross-bridges in a single pool cycle, if all cross-bridges are equivalent; or 2), the rates and fractions of total cross-bridges that cycle in each pool when there are multiple cycling pools present. Experiments were also performed under non-steady-state conditions, such as during release of catch force by phosphorylation of twitchin and during muscle shortening and lengthening. In these cases, there is the possibility of a significant redistribution of myosin among various states during the single turnover measurement. The time course of single turnover of myosin-bound ADP in these experiments may include turnover resulting from such a redistribution of myosin. For example, a perturbation that results in a fast net detachment of cross-bridges would result in high rate of turnover if it were associated with ADP release from myosin and subsequent binding and splitting of ATP. Such non-steady-state experiments thus have the potential to probe how various manipulations impact cross-bridge detachment. In summary, the single turnover technique lends itself well to the study of cross-bridge turnover in catch muscle, and is especially useful for determination of how the catch mechanism modifies myosin cross-bridge kinetics.

In the studies reported here, we find that catch muscle under isometric conditions shows both fast and slow components of myosin cross-bridge cycling that probably result from a kinetic separation of calcium-bound and calcium-free cross-bridge pools. The structure responsible for catch force maintenance at intermediate [Ca^{+2}] does not kinetically alter either the fast or slow components of cross-bridge cycling under isometric conditions. This makes it very unlikely that that the decrease in force due to phosphorylation of twitchin at submaximal [Ca^{+2}] results from a change in myosin cross-bridge kinetics as suggested by Galler and colleagues (14,20). Also, there is no measurable extra turnover of myosin-bound ADP during relaxation of catch force by phosphorylation of twitchin at pCa $>$ 8. The only effect of phosphorylation of twitchin on myosin cross-bridge turnover appears to be to a slight change in the fraction of total myosin that cycles at the ''resting'' rate at pCa $>$ 8, and a modification of the initial cross-bridge cycles after onset of shortening at subsaturating [Ca^{+2}]. The results are consistent with the idea that catch force is maintained by a thick and thin filament linkage other than the myosin cross-bridge, and that the presence or absence of the link has minimal effects on myosin cross-bridge kinetics.

METHODS

Solutions

Artificial sea water contained 10 mM KCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, 428 mM NaCl, and 10 mM *n*-[2-hydroxyethyl]piperazine-*n*′-[2-ethanesulfonic acid] (HEPES). The pH was 7.4. Rigor solutions contained 30 mM piperazine-*n*,*n*′-bis(2-ethanesulfonic acid) (PIPES), 3 mM free Mg^{2+}, 20 mM ethylene glycol-bis(-aminoethyl ether)-*n*,*n*,*n*′,*n*′-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 mM leupeptin. 1,6-diaminohexane-*n*,*n*,*n*′,*n*′-tetraacetic acid (HDTA) was added to adjust ionic strength to 200 mM. The pH was 6.8. Permeabilization solution consisted of rigor solution and 1% Triton X-100.

Relaxing solutions (pCa $>$ 8) were those to which calcium was not added. They contained 30 mM PIPES, 8 mM EGTA, 2 mM MgATP, 30 mM phosphocreatine, 3 mM free Mg^{2+}, 0.5 mM leupeptin, 1 mM DTT, and 1 mg/ml creatine kinase. HDTA was added to bring the ionic strength to 200 mM. The solution also included sucrose (10 mM), and sucrose phosphorylase (0.15 units/ml) to minimize any increase in phosphate concentration. The pH was 6.8. In experiments dealing with myosin turnover 1), during release of catch force by twitchin phosphorylation, 2), during catch force maintenance at pCa $>$ 8, and 3), following a quick release in catch, the total MgATP was 1 mM. In all of these cases, the myosin turnover rate was very slow, and the lower [ATP] allowed a higher ratio ^{3}H-ATP:^{14}C-ATP during the single turnover measurement. This provided for an increased sensitivity in determining small changes in the turnover of bound ADP.

Activating solutions were similar to the relaxing solutions with the exception that the free calcium concentration was adjusted by inclusion of CaEGTA. The total EGTA was maintained at 8 mM.

Radiolabeled ATP and ATPγS

[8-^{14}C] adenosine 5′-triphosphate (56 Ci/mol) was obtained from Amersham Biosciences (Piscataway, NJ), and [2,8-^{3}H] adenosine 5′-triphosphate: ^{3}H-ATP (35 Ci/mmol) was obtained from Perkin-Elmer (Boston, MA). ATPγS (adenosine-5′-O-(γ-thio)-triphosphate) was obtained from Calbiochem (La Jolla, CA) and purified as described by Butler et al. (11).

Synthesis and purification of caged ^{3}H-ATP

Caged [^{3}H]-ATP (P_3-1(2-nitrophenyl)ethyladenosine triphosphate) was synthesized using a modification (19) of a method described by Walker et al. (21). Specific activity of the ^{3}H in the caged ATP was ~8 Ci/mmole. Purification was accomplished by HPLC on an NH_2 column (Alltech Associates, Deerfield, IL), and the caged ATP in appropriate fractions was desalted on an Extract-Clean C_{18} column (Alltech Associates). Caged ATP was eluted using a solution containing 10% aqueous ammonium bicarbonate (100 mM) in methanol. The appropriate fractions were dried, the residue taken up in 1:1 ethanol:H_2O and stored at −76°C. HPLC analysis of the purified sample showed that ~99% of total ^{3}H was associated with fractions eluting with caged ATP.

Muscle dissection and preparation

Mytilus edulis were obtained from Anastasi's Fish Market (Philadelphia, PA) and maintained in an aquarium containing aerated filtered seawater

(Instant Ocean, Carolina Biological Supply, Burlington, NC) as previously described (4,5,10,11). The mussel shell was opened by cutting the adductor muscles, and the pedal ganglia were immediately removed to prevent excitation of the muscles (3). Muscle fiber bundles (~350 μm diameter and ~1 cm in length) were isolated from the anterior byssus retractor muscle (ABRM), mounted on holders, and incubated in aerated artificial sea water at 20°C as described by Siegman et al. (4). Muscles were incubated in permeabilization solution for 30 min, followed by three rinses (3 min each) in rigor solution. The last two rigor solutions contained CPK, sucrose, and sucrose phosphorylase. In experiments where twitchin was maintained in the unphosphorylated state, a protein kinase inhibitor of cAMP-dependent protein kinase (22) obtained from Sigma-Aldrich (St. Louis, MO) was added to the last two rigor baths at a concentration of 5 μM. All experiments were performed at 20°C.

Mechanical measurements

Muscles 8–9 mm in length were mounted on a myograph similar to that described earlier (4). Force output was measured with a DSC-6 transducer (Kistler Morse, Bothell, WA). In experiments where muscles were subjected to quick releases, the release was caused by an electromechanical relay that imposed a step change in muscle length. Isovelocity stretches after activation were imposed by a synchronous motor connected to a micrometer on the myograph by a flexible shaft cable. Muscles were stretched at ~3% L_o/s.

Flash photolysis, freezing and extraction of muscles, and analysis of nucleotides

In experiments using flash photolysis, the muscles were incubated in a solution containing caged ^{3}H-ATP (~100 μCi/ml, total [caged ATP] = 12 μM) and ^{14}C-ATP (50 μCi/ml, total [MgATP] = 2 mM). A xenon flash lamp (Gert Rapp, Hamburg, Germany) with a UG11 filter provided an ultraviolet flash that released ^{3}H-ATP from caged ^{3}H-ATP. Approximately 70% of the tritium in caged ATP appeared in ATP after photolysis, changing the ratio of ^{3}H:^{14}C in ATP from almost zero before the flash to ~1.7 after the flash. The increase in [ATP] with photolysis was ~8 μM or <0.5% of total [ATP]. Muscles were frozen at liquid nitrogen temperatures either with a freeze-clamp device (model No. 669 Cryosnapper, Gatan, Warrendale, PA) or by direct immersion into a vial containing liquid nitrogen (for muscles frozen at times of ≥3 s). The timing of the flash lamp, changes in muscle length, and the freeze-clamp device were under computer control. The fraction of ADP exchanged was determined by comparing the ratio ^{3}H:^{14}C in ADP to that in ATP. Nucleotides were extracted from the frozen muscles and separated by HPLC on either an Econosil NH_2 10 μm or an α-bond NH_2 10 μm column (Alltech Associates). The mobile phase gradients were adjusted to give appropriate separation of the nucleotides of interest (11). The column effluent was collected, and the radioactivity associated with each fraction was measured. Vyas et al. (19) reported that photolysis on an inert matrix of solutions containing caged ^{3}H-ATP and ^{14}C-ATP causes a small quantity of ^{3}H and ^{14}C to appear in the ADP fraction. The amount of ^{3}H and ^{14}C that appears in ADP independent of ADP turnover in the muscle is dependent upon the solution and was measured in every experiment. This background was subtracted from every sample in each experiment. In some cases noted in Results, the single turnover protocol was initiated by transfer of the muscle from a solution containing ^{14}C-ATP to a solution containing ^{14}C-ATP and ^{3}H-ATP. In these cases, the specific activity of ^{14}C-ATP was similar to that described above, and the specific activity of ^{3}H-ATP was 2–4 times that of the ^{14}CATP.

Phosphorylation and thiophosphorylation of twitchin

In permeabilized muscle, twitchin was phosphorylated by the addition of 100 μM cAMP in the presence of MgATP (4,10,11). The thiophosphorylation of twitchin was accomplished by adding ATPγS (100 μM) to permeabilized muscle in the presence of cAMP for 10 min (4,10,11). This was followed by three washes in rigor solution for 10 min each. Thiophosphorylated twitchin is not susceptible to dephosphorylation and has the same mechanical effect on catch force as does phosphorylation (4,10).

Statistics

All data are reported as mean ± SE. In all comparisons, $P < 0.05$ is considered to represent a significant difference. Statistical analysis was performed using either the student's *t*-test or ANOVA. Regression analysis was performed using SigmaPlot software (Systat, San Jose, CA).

Simulations

The SCoP software package (Simulation Resources, Redlands, CA) was used to generate simulations of kinetic models.

RESULTS

Time course of the single turnover of myosin-bound ADP as a function of [Ca^{2+}]

The time course of the single turnover of myosin-bound ADP in permeabilized ABRM was determined during steady-state force maintenance at various [Ca^{2+}] when twitchin was unphosphorylated. The experimental design is shown in Fig. 1 *A*. After treatment in rigor solution, muscles were incubated in a solution containing ^{14}C-ATP (Total ATP = 2 mM) and high specific activity caged ^{3}H-ATP. This procedure allows for initial labeling of myosin with ^{14}C-ADP. Free [ADP] was kept very low by the presence of phosphocreatine (30 mM) and creatine phosphokinase (1 mg/ml). UV flash photolysis released ^{3}H-ATP from caged ^{3}H-ATP with only a minimal change in the total [ATP]. ATP that subsequently binds to myosin has effectively the same specific activity of ^{14}C as before the UV flash, but there is now also ^{3}H-ATP present. In this way, the myosin-bound ADP that results from the subsequent splitting of the ATP acquires the tritium label. Muscles were frozen at various times after the flash, and the radioactivity of ADP, ATP, and caged ATP was measured. The fraction of bound ADP exchanged was determined by comparing the ratio ^{3}H:^{14}C in ADP to that in ATP.

The time courses of the single turnover of myosin-bound ADP were determined at pCa > 8, 6.3, 6.0, and 5.3 where isometric force output was 0, 29, 91, and 100% of maximum, respectively. The results are shown in Fig. 1 *B*. At pCa > 8, the single turnover of myosin-bound ADP is very slow, and there are increasingly faster time courses as [Ca^{2+}] is increased. The results also show that the maximum myosin-bound ADP exchange is ~60%. It is not known why almost 40% of the myosin does not exchange its ADP in this experimental design. A similar lack of complete exchange of myosin-bound ADP was seen in permeabilized mammalian smooth muscle with fully thiophosphorylated light chains (19). It might be that only one head of the double-headed

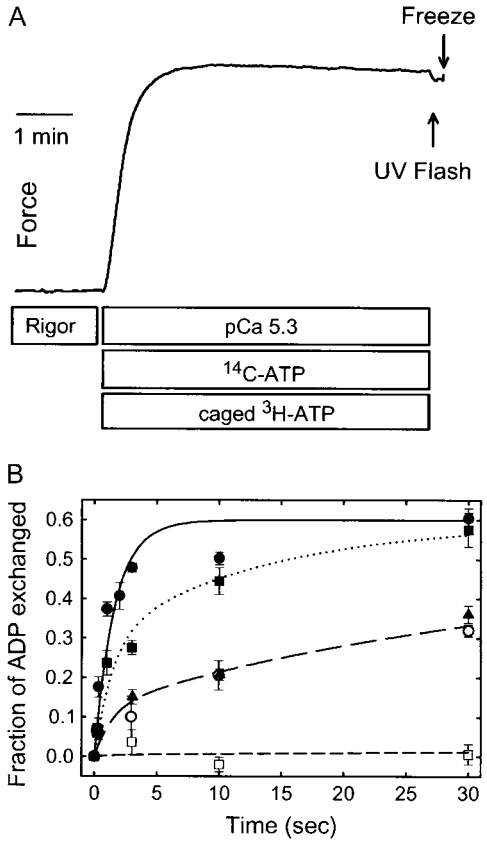

FIGURE 1 Single turnover of myosin-bound ADP under isometric conditions in permeabilized ABRM as a function of [Ca^{+2}]. (*A*) Experimental design and a typical force trace at pCa 5.3; muscles were frozen at various times after the flash. (*B*) Time course of ADP turnover after flash photolysis in the absence of cAMP, pCa 5.3 (*solid circles*), pCa 6 (*solid squares*), pCa 6.3 (*solid triangles*), pCa $>$ 8 (*open squares*), and at pCa 6.3 in the presence of cAMP (*open circles*). The lines are simulations from a four-state model described by Butler et al. (10) in which the percent of calcium-bound myosin was 92 (*solid line*), 55 (*dotted line*), 23 (*long dashed line*), and 1 (*short dashed line*). A maximum exchange of 0.6 was assumed. See Discussion for more detail. Data are mean $\pm$ SE, $N = 3$–22.

myosin can be activated in both of these smooth muscles. It is also possible that within each muscle access of different myosin molecules to actin may vary because of structural arrangements of the molecules in the filaments.

At pCa 5.3, force is maximum, and myosin is likely to be nearly saturated with calcium. At this [Ca^{2+}], the ADP single turnover data are consistent with an exponential that has a rate constant of $\sim$1 s^{-1}. This rate constant probably reflects that of the calcium-bound, fast-cycling cross-bridge. Total bound ADP is 108 $\pm$ 6 μM, and since 60% of this turns over at a rate constant of $\sim$1 s^{-1}, the overall myosin ATPase at pCa 5.3 would be $\sim$65 μM/s. This value is similar to that obtained previously by measurement of steady-state ATPase activity (10). At pCa 6.0 and 6.3, both fast and slow components of ADP single turnover are apparent, and the fraction of the total associated with the fast component decreases as [Ca^{+2}] is lowered.

Effect of twitchin phosphorylation on single turnover of myosin-bound ADP at subsaturating [Ca^{+2}]

The cAMP-dependent phosphorylation of twitchin has been shown to cause a significant reduction in force at subsaturating [Ca^{2+}] (5). The change in force is not accompanied by a measurable change in muscle ATPase activity (10). Such experiments have been interpreted to mean that catch links can maintain force at subsaturating, but suprabasal [Ca^{+2}] without a significant energy input. To directly test the effect of removal of catch links on the kinetics of myosin cross-bridge cycling, the effect of phosphorylation of twitchin on the single turnover of myosin bound ADP was determined at pCa 6.3. The design of these experiments was similar to that shown in Fig. 1 *A* except that the presence of cAMP resulted in phosphorylation of twitchin. Other experiments showed that under these conditions, the cAMP-mediated phosphorylation of twitchin caused a 22 $\pm$ 6% decrease in isometric force. The data are shown in Fig. 1 *B*. There is no significant difference in the time course of the single turnover of ADP at pCa 6.3 when twitchin is phosphorylated and catch force is not present, as compared to when twitchin is unphosphorylated and catch force is present. These data support the view that the removal of catch links has no detectable effect on the kinetic parameters of the cycling myosin cross-bridges at subsaturating [Ca^{+2}].

Myosin-bound ADP turnover during catch force maintenance

The myosin-bound ADP turnover during catch force maintenance was determined in an experimental design shown in Fig. 2 *A*. Muscles were maximally activated (pCa 5) in a solution containing ^{14}C-ATP, then transferred to a pCa $>$ 8 solution containing ^{14}C-ATP for 2.5 min. During this time, calcium-activated cross-bridge cycling stops, and the muscle makes the transition into the catch state. The single turnover experiment is then initiated by transferring the muscle to a solution containing both ^{3}H-ATP and ^{14}C-ATP. The extent of turnover of myosin-bound ADP was determined as described earlier. The expected slow turnover of myosin-bound ADP under these conditions obviated the need for introduction of the ^{3}H-ATP by flash photolysis. The results (Fig. 2 *B*), indeed, show a very slow time course of myosin-bound ADP exchange. A single exponential fit to the data shows a maximum exchange of 0.55, which is similar to that under maximum activation (see Fig. 1 *B*), and a rate constant of 0.0043 s^{-1}, which is $\sim$200-fold slower.

The myosin-bound ADP turnover during relaxed conditions (i.e., in the absence of catch force maintenance) was determined in a similar design in which twitchin was thiophosphorylated (Fig. 2 *A*). Under these conditions, catch force is not maintained after the transition from pCa 5 to pCa $>$ 8. The results are shown in Fig. 2 *B*. There is a significantly ($P < 0.002$) lower fraction of ADP exchanged as a function of time when the muscle is relaxed (twitchin

thiophosphorylated) compared to when catch force is maintained (twitchin unphosphorylated). An exponential fit to the data from the relaxed muscle shows a similar rate constant to that in catch (0.0046 s^{-1}, relaxed versus 0.0043 s^{-1}, catch), but there is a small but significant ($P < 0.002$) decrease in the maximum extent of exchange in relaxed (0.47 ± 0.02) compared to catch (0.55 ± 0.02) conditions. These data suggest that catch force maintenance is associated with a small and very slow extra turnover of myosin compared to the relaxed state.

Is there a large turnover of myosin-bound ADP during relaxation of catch force by twitchin phosphorylation?

We have previously suggested that the catch force link is the calcium-free myosin cross-bridge attached to actin (10,11).

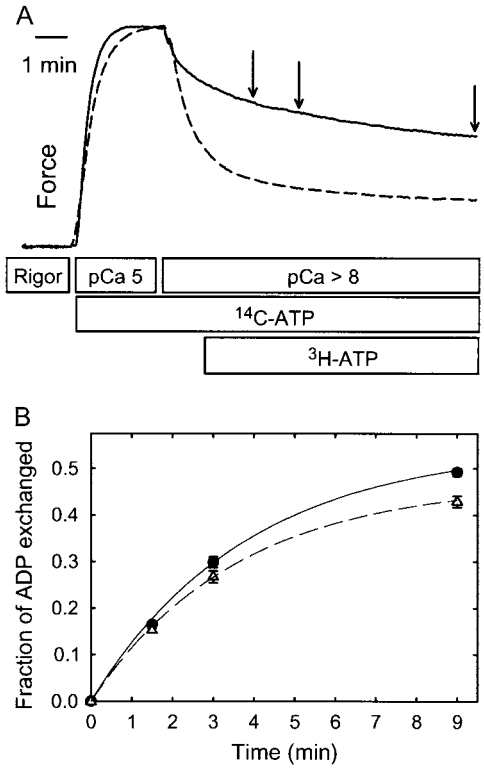

FIGURE 2 (*A*) Experimental design and typical force traces for determination of the single turnover of myosin-bound ADP during catch (*solid line*) compared to that when twitchin is thiophosphorylated, and no catch force is present (*dashed line*). Thiophosphorylation of twitchin was accomplished by incubation of the muscles in cAMP and ATPγS before the start of experiment. The single turnover protocol was initiated by the addition of ^{3}H-ATP. Muscles were frozen at times indicated by the arrows. (*B*) Time course of the single turnover of myosin-bound ADP during catch force maintenance (*solid circles*, *solid line*) and when no catch force is present due to thiophosphorylation of twitchin (*open triangles*, *dashed line*). Lines are single exponentials showing the best fit for the data. The rate constant and maximum exchange when twitchin is unphosphorylated are 0.26 min^{-1} and 0.55, respectively; and when twitchin is thiophosphorylated, they are 0.28 min^{-1} and 0.47, respectively. Data are mean ± SE, $N = 3$–7.

Some of the evidence supporting this hypothesis was the observation that there was an increase in the turnover of myosin-bound ADP with twitchin phosphorylation-mediated relaxation of catch (11). This was consistent with the idea that phosphorylation of twitchin promoted ADP release from the catch cross-bridge and subsequent detachment of the cross-bridge from actin after ATP binding. In these previous studies, the experimental design did not allow for the determination of the fraction of total myosin that turned over ADP with relaxation of catch. Rather, the design was such that small changes in ADP turnover would be magnified. More recent studies have provided strong evidence against the high force myosin cross-bridge being the catch-force maintaining link (15). It was, therefore, of interest to perform experiments to directly measure the fraction of myosin that turns over its ADP with the relaxation of catch by twitchin phosphorylation. The design of the experiment is shown in Fig. 3 *A*. Muscles were put into the catch state in the presence of ^{14}C-ATP as described earlier, and the single turnover protocol was initiated with the addition of ^{3}H-ATP along with the ^{14}C-ATP. Ninety seconds later, cAMP was added to the solution to result in twitchin phosphorylation and relaxation of catch force. Muscles were frozen at various times during the time course of relaxation of catch force and the radioactivity in ADP determined. Control muscles were treated identically except that no cAMP was added. Importantly, the presence of a constant specific activity ^{14}C-ATP allowed the measurement of the fraction of total myosin that turned over ADP with relaxation of catch compared to the control muscles in which catch force was maintained.

The results (Fig. 3 *B*) show no significant increase in myosin-bound ADP exchange in muscles during the transition from catch to relaxation of catch compared to controls. This means that the detachment of catch force-maintaining links is not associated with ADP turnover on a major fraction of myosin.

Myosin kinetics during muscle shortening and lengthening

Strain dependence of ADP release from the actomyosin complex has become a central tenet in the mechanism of myosin cross-bridge function. The next series of experiments determined how the kinetics of myosin-bound ADP turnover in catch muscle changes in response to mechanical perturbations that alter the strain of the cross-bridge. At saturating [Ca^{+2}], there is no evidence of the presence of catch force-maintaining links. On the other hand, at subsaturating [Ca^{+2}], some catch force is present when twitchin is unphosphorylated, but it is relaxed with no change in ATPase activity when twitchin is phosphorylated. It was therefore possible to test how the presence or absence of catch force maintaining links affects the strain dependence of myosin cross-bridge turnover.

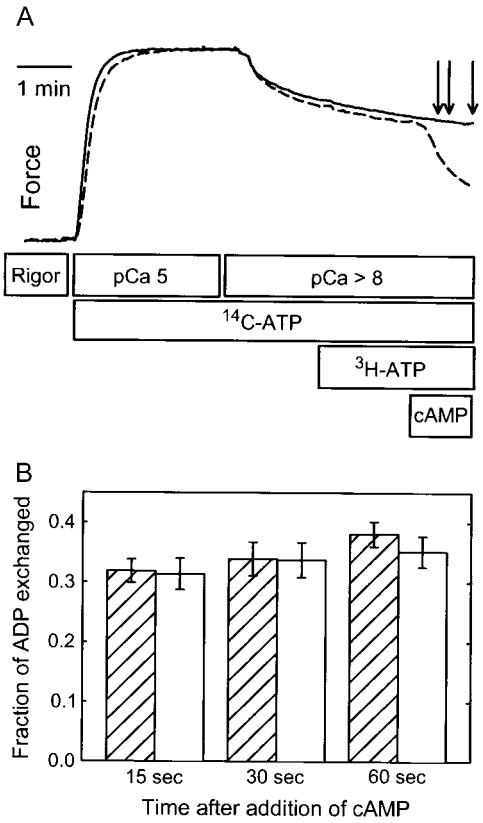

FIGURE 3 Single turnover of myosin-bound ADP associated with relaxation of catch force by phosphorylation of twitchin. (*A*) Experimental design and typical force traces. After catch force was developed, it was either maintained (*solid line*) or relaxed by addition of cAMP and phosphorylation of twitchin (*dashed line*). The single turnover protocol was initiated by the addition of ^{3}H-ATP, and muscles were frozen at times indicated by the arrows. (*B*) Fraction of myosin-bound ADP exchanged at various times during catch force maintenance (*hatched bars*) and during the relaxation of catch force subsequent to the addition of cAMP (*open bars*). Data are mean ± SE, $N = 6$–9.

Effect of unloaded shortening on the single turnover of myosin-bound ADP at saturating [Ca^{+2}]

In the experimental design for determining the effect of unloaded shortening on myosin-bound ADP turnover, muscles were activated at pCa 5.3 in a solution containing ^{14}C-ATP and caged ^{3}H-ATP, followed by UV flash photolysis. The length of the experimental muscles was then quickly decreased (<10 ms) by ~25% L_o. During the measurement period (330 ms) of the single turnover, there was no force redevelopment, indicating that the muscles were slack and shortening was occurring under unloaded conditions. Isometric control muscles were treated identically except that their length was unchanged. The results (Fig. 4) show a significantly ($P < 0.0002$) faster time course of ADP exchange when muscles are released and allowed to shorten compared to muscles that remain isometric. The extent of the increase in rate constant for exchange is approximately threefold, as shown by the curves with rate

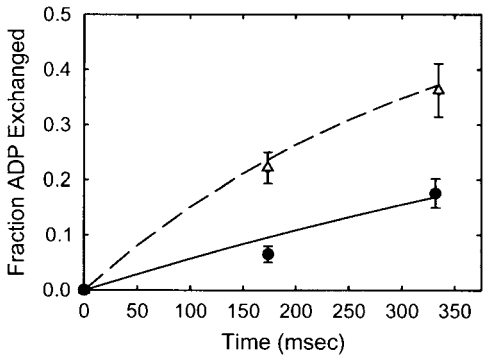

FIGURE 4 Effect of shortening on single turnover of myosin-bound ADP at pCa 5.3. Muscles were incubated in ^{14}C-ATP and caged ^{3}H-ATP for 3 min at pCa 5.3. They were then exposed to a UV flash and after a 10 ms delay subjected to a quick release of ~25% L_o. Control muscles remained isometric. Shown is the time course of the single turnover of myosin-bound ADP under isometric (*solid circle*) and shortening (*open triangle*) conditions. The lines are exponentials assuming a maximum exchange of 0.55 with rate constants of 1 s^{-1} (*solid line*) and 3 s^{-1} (*dashed line*). Data are mean ± SE, $N = 4$–5.

constants of 1 s^{-1} (isometric) and 2.9 s^{-1} (shortening) assuming a maximum exchange of 0.55.

Effect of stretch during activation on the single turnover of myosin-bound ADP

Maximum force was allowed to develop at pCa 5.3 in a solution containing ^{14}C-ATP and caged ^{3}H-ATP, and the muscles were then subjected to a 3% L_o/s stretch started 0.1 s before the UV flash that initiated the single turnover protocol (Fig. 5 *A*). Isometric control muscles were treated identically except for the stretch. The results (Fig. 5 *B*) show no significant effect of the stretch on the time course of ADP turnover compared to isometric conditions. Therefore, in the maximally activated muscle, there is a strain-dependent increase in the rate of myosin-bound ADP turnover when filaments slide in the shortening direction, but not an observable decrease in turnover rate when cross-bridges are strained in the lengthening direction.

Effect of quick release on the single turnover of myosin-bound ADP during catch

To determine whether there is a strain dependence of ADP turnover on myosin during catch, muscles in catch were subjected to a quick release during a single turnover protocol. The design and a typical force trace are shown in Fig. 6 *A*. Control muscles were not released. There is no significant difference in the fraction of ADP exchanged when muscles were released during catch as compared to isometric controls (Fig. 6 *B*). These results suggest that during catch, cross-bridges do not show an increase in the rate constant for ADP release when force is decreased to zero as a result of a decrease in muscle length.

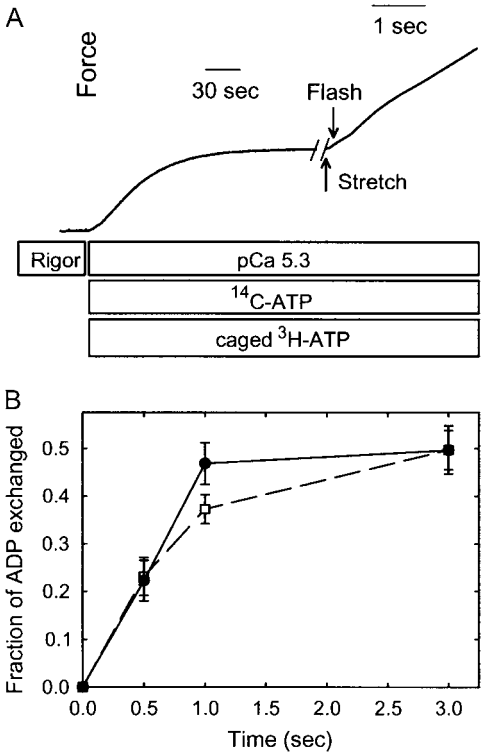

FIGURE 5 Single turnover of myosin-bound ADP during stretch. (*A*) Experimental design and typical force trace for a muscle in which the single turnover of bound ADP was measured during an isovelocity stretch at 3% L_o/s. The stretch was initiated 100 ms before photolysis. Control muscles were treated similarly, but remained isometric. Muscles were frozen at different times after photolysis. (*B*) Time course of the single turnover of myosin-bound ADP during isovelocity stretches (*open squares*, *dashed line*) and during isometric contractions (*solid circles*, *solid line*). Data are mean ± SE, $N = 4$–5.

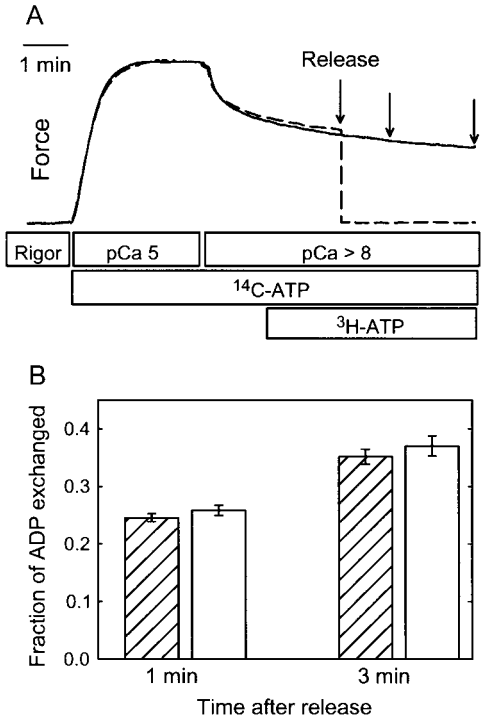

FIGURE 6 Single turnover of myosin-bound ADP after a quick release of a muscle in catch. (*A*) Experimental design and typical force traces in muscles subjected to a quick release of ~10% L_o (*dashed line*) while in the catch state. Control muscles remained isometric (*solid line*). The single turnover protocol was initiated by the addition of ^{3}H-ATP as shown, and the muscles were frozen at the times indicated by the arrows. (*B*) Fraction of myosin-bound ADP exchanged at different times during isometric conditions (*hatched bars*), and after the quick release (*open bars*). Data are mean ± SE, $N = 8$.

Strain dependence of myosin-bound ADP turnover at intermediate [Ca^{+2}]

There is an increase in the time course of the single turnover of ADP at saturating [Ca^{+2}] when muscles undergo unloaded shortening, but not when muscles in catch are subjected to a quick release. This suggests that calcium-bound cycling myosin cross-bridges exhibit strain-dependent turnover, whereas catch-force maintaining links do not. At intermediate [Ca^{+2}] both of these force-maintaining processes are present when twitchin is unphosphorylated, but catch links are removed by twitchin phosphorylation. It is, therefore, possible to test the effect of catch force links on the strain dependence of myosin cross-bridge turnover by determining the strain dependence of myosin-bound ADP turnover at intermediate [Ca^{+2}] as a function of twitchin phosphorylation.

The experiment was similar to that described in Fig. 4, except that the muscles were activated at pCa 6. The results (Fig. 7) show that when twitchin is unphosphorylated, there is no significant change in the fraction of ADP exchanged during an unloaded shortening compared to isometric conditions. This is in sharp contrast to the large strain dependence seen at saturating [Ca^{+2}] (Fig. 4) when no catch force maintenance occurs. These results suggest that the presence of catch links modifies the strain dependence of myosin-bound ADP turnover. This idea was tested directly by measuring ADP turnover at pCa 6 in the presence of cAMP, which leads to phosphorylation of twitchin. The results from this experiment (Fig. 8) show a significant ($P < 0.05$) increase in the fraction of ADP exchanged when muscles are released as compared to isometric control muscles. The results suggest that catch links may place a structural constraint on calcium-bound cross-bridges, and, thereby interfere with the ability of the calcium-bound cross-bridge to increase the ADP off rate during muscle shortening. If so, this constraint is removed when twitchin is phosphorylated.

DISCUSSION

Myosin kinetics as a function of [Ca^{+2}]

At suprabasal, but subsaturating calcium concentrations, there is evidence of both fast and slow components of myosin-bound ADP turnover under isometric conditions. The fast component is a greater fraction of the total at higher

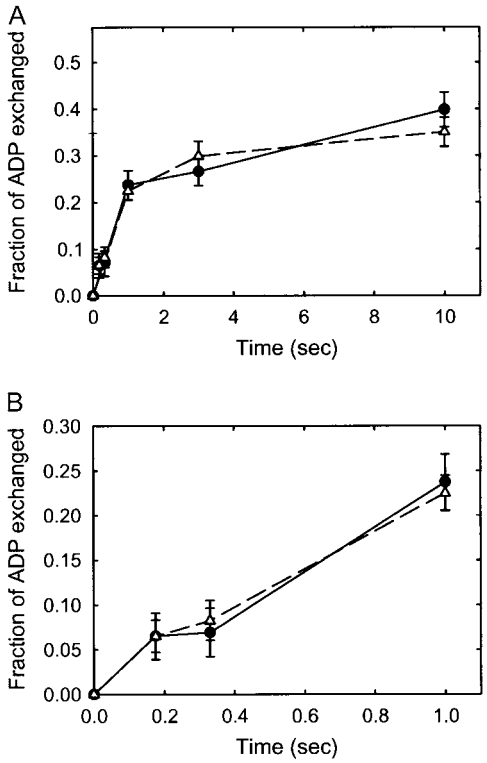

FIGURE 7 Time course of the single turnover of myosin-bound ADP at pCa 6 during unloaded shortening and during isometric conditions. The experimental design is similar to that shown in Fig. 4 except for a lower [Ca^{+2}]. (*A*) Fraction of ADP exchanged as a function of time after the UV flash for muscles subjected to unloaded shortenings (*open triangles*, *dashed line*) and isometric contractions (*solid circles*, *solid line*). (*B*) The same data on an expanded timescale. Data are mean ± SE, $N = 4$–6.

[Ca^{+2}], and it is probably associated with those cross-bridges that have calcium bound at the start of the single turnover protocol. The slow component may represent those cross-bridges that must subsequently bind calcium to enter into the fast cycling cross-bridge pool. In such a model, the turnover

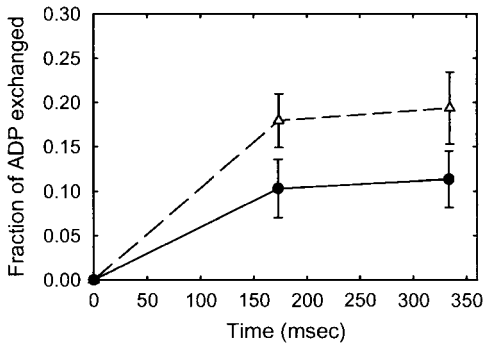

FIGURE 8 Time course of the single turnover of myosin-bound ADP at pCa 6 during unloaded shortening (*open triangles*, *dashed line*) and during isometric conditions (*solid circles*, *solid line*) when twitchin is phosphorylated. The experimental design is similar to that described in Fig. 7 except that cAMP is included in all solutions to ensure phosphorylation of twitchin. Data are mean ± SE, $N = 6$–8.

of calcium on the cross-bridge would have to be slow compared to the cross-bridge cycle to maintain substantial kinetic separation of the calcium-bound and calcium-free cross-bridge pools. The time course of myosin-bound ADP turnover is not significantly changed by the presence or absence of catch force at pCa 6.3, suggesting that the structure responsible for catch force maintenance does not participate in or kinetically alter those mechanisms responsible for the fast and slow components. This makes it very unlikely that that the decrease in force due to phosphorylation of twitchin at submaximal [Ca^{+2}] results from a change in myosin cross-bridge kinetics as suggested by Galler and colleagues (14,20).

The fraction of the total myosin-bound ADP exchange that is part of the fast component is substantially smaller in pCa 6 compared to pCa 5.3 (see Fig. 1 *B*), while the force output is only ~10% lower. Previous experiments also showed that there is a much lower ATPase activity per unit of force output as [Ca^{+2}] decreases (10). While some of this high force output with low ATPase is likely due to the presence of catch-force maintaining structures at intermediate [Ca^{+2}], variation in the energy cost of force output is observed even when twitchin is phosphorylated, and catch force is relaxed. These results suggest that the number of fast cycling cross-bridges is not the sole determinant of isometric force output in the absence of catch force. It is possible that calcium-free myosin cross-bridges contribute to force output through a cooperative activation mechanism as has been described for mammalian smooth muscle (18,19) or perhaps as a result of slow detachment of cross-bridges that unbind calcium while in the high-force, actin-bound state. The latter mechanism is similar to the proposal for latchbridge formation by dephosphorylation of attached myosin cross-bridges in mammalian smooth muscle (23). It is interesting that the invertebrate catch smooth muscle may, when not in the catch state, show some aspects of the latch state normally associated with vertebrate smooth muscle.

The observed time courses of single turnover of myosin-bound ADP in the ABRM can be simulated by a simple four-state model of the cross-bridge cycle that we previously proposed for catch muscle (10). This model was developed to simulate the relationships between force and ATPase activity at different [Ca^{+2}]. The four states include detached (no force) and attached (force-producing) cross-bridges each with or without calcium bound. In this model, the rate constant for calcium unbinding from both detached and attached cross-bridges is relatively slow (0.05 s^{-1}). The transition from detached to attached calcium-bound cross-bridge has a rate constant of 3 s^{-1} while calcium-free cross-bridges cannot directly make the transition to attached calcium-free cross-bridges. Calcium-free attached cross-bridges can be formed by calcium unbinding from the attached calcium-bound species. Calcium-bound cross-bridges detach more rapidly than calcium-free attached cross-bridges (0.8 vs. 0.06 s^{-1}). The lines in Fig. 1 *B* show time courses of myosin-bound ADP exchange calculated from the model when the percent

of myosin with calcium bound is 1, 23, 55, and 92, which fit the data for pCa $>$ 8, 6.3, 6.0, and 5.3, respectively. The simulations support the idea that the slow and fast components of the time courses of single turnover of myosin-bound ADP result from kinetic separation of the calcium-bound and calcium-free cross-bridge pools.

In the original version of this model, the detachment rate constant for the calcium-free attached cross-bridge was controlled by the phosphorylation state of twitchin. This was based on the idea that the catch force link is the calcium-free, actin-attached myosin cross-bridge that has a very slow rate constant (0.006 s^{-1}) for detachment when twitchin is unphosphorylated. It was suggested that twitchin phosphorylation increased this rate constant by 10-fold. In such a model under steady-state conditions, the phosphorylation state of twitchin has a minimal effect on the simulation of the time course of ADP turnover at a given [Ca^{+2}]. However, during the relaxation of catch force at low [Ca^{+2}] by twitchin phosphorylation, there would be burst of ADP turnover associated with catch cross-bridge detachment. The amount of ADP turnover would be determined by the fraction of myosin attached to actin and maintaining catch force. The results in Fig. 3 show no significant change in the single turnover of myosin-bound ADP during the relaxation of catch force by twitchin phosphorylation. This argues against the idea that twitchin phosphorylation controls the rate constant for ADP release from and detachment of a large fraction of the calcium-free myosin. While this is consistent with the recent view that catch force is maintained by a structure other than the myosin cross-bridge (13–15,24), it is possible that a very small fraction of myosin whose turnover is not detectable in such an experiment may maintain catch force under these conditions.

During catch force maintenance, the myosin-bound ADP turnover is ~200-fold slower than that during maximal activation. Indeed, the rate constant for the turnover during catch (twitchin unphosphorylated, pCa $>$ 8) is very similar to that measured under relaxed conditions (twitchin phosphorylated, pCa $>$ 8). But, there is a small extra fraction of myosin that turns over at this relaxed rate when catch force is maintained. This is consistent with previous observations that there is a small extra myosin-bound ADP exchange and suprabasal ATPase associated with catch force maintenance (10,11,25). The basis of the extra myosin in this very slow cycling pool during catch is not known. But, the structures that maintain catch force may bring a small portion of inactive myosin into a structural configuration that is more typical of the myosin responsible for the major portion of the resting myosin-bound ADP turnover at pCa $>$ 8. This could result from a subtle change in thick filament structure such as modification of the interaction of the cross-bridge with the thick filament backbone, and/or an increase in the probability that the cross-bridge could interact with actin.

It is interesting to compare changes in the relationship between force output and cross-bridge cycling rate in invertebrate smooth muscle which shows catch, and in vertebrate smooth muscle which shows latch. In intact mammalian smooth muscles, there is an approximately fourfold increase in the economy (force/ATPase) of force maintenance as myosin light chain phosphorylation goes from high to low levels during activation (26–28). In intact catch muscle, there is an 11-fold increase in economy as the muscle makes the transition from the initial phasic to the tonic catch-force maintaining segment of the contraction (25). Since total ATPase potentially includes many processes other than myosin cross-bridge cycling, a far better estimate of the change in the relationship between myosin turnover and force output can be obtained from data on single turnover of myosin-bound ADP. These data show a 200-fold decrease in turnover during catch compared to maximal activation. Force output during catch is 25–50% of maximum force, so there is a 50-to-100-fold higher economy during catch. The turnover of the myosin cross-bridge during latch in vertebrate smooth muscle has not been measured directly, but Vyas et al (19) reported that the fraction of myosin showing fast turnover of myosin-bound ADP is directly proportional to the extent of myosin light chain phosphorylation. Therefore, the extent of phosphorylation can be used as a rough estimate of the myosin turnover rate. If myosin light chain phosphorylation decreases from 60% during initial activation to ~20% during latch, as found in the swine carotid artery with K^+ depolarization (29), then there would be an approximately threefold decrease in ATPase; if force output was unchanged, there would be a threefold increase in economy. The extreme change in economy of the catch muscle (~50-fold) compared to mammalian smooth muscle (approximately threefold) is because myosin cross-bridge cycling is almost completely shut down during catch force maintenance. In contrast, there is always a finite degree of myosin light chain phosphorylation in mammalian smooth muscle during latch, and this leads to some fast myosin cross-bridge cycling. The mechanism for force maintenance in latch may very well be driven by a small amount of fast cycling myosin, while the evidence strongly suggests that this is not the case in catch force maintenance.

Myosin turnover during shortening

More than 80 years ago, Fenn (30) reported that muscles liberated energy at a higher rate while shortening than when length was held constant under isometric conditions. This observation conclusively showed that the rates of the reactions responsible for energy utilization in muscle varied with changes in load. Huxley (31) incorporated such findings into a cross-bridge model of muscle contraction by postulating that the rates of cross-bridge attachment and/or detachment depend on the strain of the cross-bridge. The identity of the strain-dependent step(s) in the cross-bridge cycle is not certain, but it has long been thought that the rate of ADP release could change as a function of load (see (32)

for review). In some types of myosin (including smooth muscle) there is evidence that an extra movement of the myosin head is associated with ADP release (33,34). High force isometric conditions would inhibit such movement, while low force shortening conditions would facilitate the movement and increase the rate of ADP release from myosin (32,35,36).

The results reported here for maximally activated muscles are consistent with an approximately threefold increase in myosin-bound ADP turnover when the cross-bridge is unstrained as a result of a quick release. The use of a similar design showed an approximately fivefold increase in myosin-bound ADP turnover when activated mammalian smooth muscle was subjected to a quick release (37). Also, Khromov et al. (38), using a different method, found that a change in external strain caused an approximately twofold change in the rate constant for ADP release from both unphosphorylated and thiophosphorylated myosin in mammalian smooth muscle. Thus, the fully activated catch muscle shows a very similar strain-dependent change in cross-bridge turnover, as do other smooth muscles. This suggests that under fully activated conditions, the catch mechanism present in this muscle does not have a large effect on the factors responsible for the increase in cross-bridge turnover during shortening. This is consistent with the idea that the structure responsible for catch force does not play a significant mechanical role when the muscle is maximally activated (15).

The results obtained when the muscle is partially activated suggest a very different scenario. In this case, no significant increase in the myosin-bound ADP turnover could be demonstrated after the muscle was subjected to a quick release when twitchin was unphosphorylated. However, an increase in cross-bridge turnover is measurable when twitchin is phosphorylated, and the catch component of force is relaxed. Clearly, the catch structure alters the response of myosin to a decrease in load on the muscle. The catch structure may prevent the immediate motion of the myosin head associated with unloading of the muscle. This could occur if the catch structure tightly links thick and thin filaments and delays the relative movement of the myosin heads after unloading of the muscle. There could also be dissociation between motion of the myosin head and an increase in the rate of ADP release. However, this would probably require direct interaction of each myosin head with twitchin; and given the relative contents, each twitchin would have to control 13 double-headed myosin molecules.

It is somewhat surprising that at intermediate [Ca^{+2}] there is a large effect of twitchin phosphorylation on the strain dependence of single turnover of myosin-bound ADP, but not on unloaded shortening velocity (V_o) (5,10). In addition, the fact that there is active shortening of the muscle at intermediate [Ca^{+2}] when twitchin is unphosphorylated strongly indicates that unloading the muscle under these conditions does indeed lead to the changes in cross-bridge kinetics associated with shortening of the muscle. It is possible that the mechanical constraint presented by catch linkages affects only the initial cross-bridge cycle after the onset of shortening. During a slack test to determine V_o, many rounds of cross-bridge cycling occur, and the effect of the catch linkage may diminish with time after release of the muscle. This would allow the catch structure to have a relatively large effect on observations dealing with the first cross-bridge cycles (such as single turnovers), but not on those that are associated with many cycles (such as V_o determinations).

Myosin cross-bridge turnover during stretch

Stretching skeletal muscles during activation causes a decrease in total energy output (39) resulting from a suppression of the actin-activated myosin ATPase (40). It is thought that stretch inhibits energy usage compared to isometric conditions because the very high force makes it less probable that myosin can assume the conformation in which ADP release can occur. However, the results presented here show no significant effect of stretch on the single turnover of myosin bound ADP in catch muscle. Butler et al. (41) found a rather complicated effect of stretch during activation on high energy phosphate usage in mammalian smooth muscle. Compared to isometric controls, they found a decrease in energy usage when muscles were stretched during force development when isometric energy usage was high, but no change when stretch occurred during the force maintenance phase when isometric energy usage was low. It seems that when the energy usage is already low during constant force maintenance under isometric conditions, stretching smooth muscle does not drive it lower. It is possible that during high force output under isometric conditions, the extra movement of the myosin head that facilitates ADP release as described earlier might be maximally inhibited. In other words, the ADP release rate under constant force isometric conditions may be the low value associated with the conformation of myosin after the first of the two possible lever arm movements. If this were the case, then the large increase in cross-bridge strain associated with stretch during activation would have little or no effect on the time course of myosin-bound ADP compared to isometric conditions.

SUMMARY

In summary, catch muscle shows fast and slow components of cross-bridge cycling that probably result from a kinetic separation of calcium-bound and calcium-free cross-bridge pools. Even with the catch component removed, the force output at low [Ca^{+2}] is larger than expected from the fraction of fast cycling myosin cross-bridges, suggesting that slow cycling, calcium-free cross-bridges contribute to force output. The structure responsible for catch force maintenance at intermediate [Ca^{+2}] does not participate in or kinetically alter the processes responsible for the fast and slow components under isometric conditions. Also, there is no measurable

turnover of myosin-bound ADP during relaxation of catch force by phosphorylation of twitchin at pCa > 8. Indeed, the only effects of the catch link on myosin-bound ADP turnover are 1), a small, very slow extra turnover when catch force is maintained at very low [Ca^{+2}] (pCa > 8); and 2), attenuation of the shortening-induced increase in turnover at subsaturating [Ca^{+2}]. These limited interactions between the catch link and myosin cross-bridge turnover are consistent with the idea that catch force is maintained by a thick and thin filament linkage other than the myosin cross-bridge. The linker may be twitchin itself, and its interaction with the thin filament might be controlled by both its state of phosphorylation and by the myosin cross-bridge (15). But, conversely, the twitchin phosphorylation-mediated control of myosin cross-bridge cycling appears to be limited to a slight increase in the fraction of total myosin that cycles at the resting rate at pCa > 8, and an inhibition of at least the initial cross-bridge cycles after onset of shortening at subsaturating [Ca^{+2}].

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REFERENCES

1. Ishii, N., A. W. M. Simpson, and C. C. Ashley. 1989. Free calcium at rest during ''catch'' in single smooth muscle cells. *Science*. 243:1367–1368.
2. Twarog, B. M. 1967. The regulation of catch in molluscan muscle. *J. Gen. Physiol.* 50:157–169.
3. Cole, R. A., and B. M. Twarog. 1972. Relaxation of catch in a molluscan smooth muscle. I. Effects of drugs which act on the adenyl cyclase system. *Comp. Biochem. Physiol.* 43:321–330.
4. Siegman, M. J., S. U. Mooers, C. Li, S. Narayan, L. Trinkle-Mulcahy, S. Watabe, D. J. Hartshorne, and T. M. Butler. 1997. Phosphorylation of a high molecular weight (approximately 600 kDa) protein regulates catch in invertebrate smooth muscle. *J. Muscle Res. Cell Motil.* 18:655–670.
5. Siegman, M. J., D. Funabara, S. Kinoshita, S. Watabe, D. J. Hartshorne, and T. M. Butler. 1998. Phosphorylation of a twitchin-related protein controls catch and calcium sensitivity of force production in invertebrate smooth muscle. *Proc. Natl. Acad. Sci. USA*. 95:5383–5388.
6. Szent-Gyorgyi, A. G. 1996. Regulation of contraction by calcium binding myosins. *Biophys. Chem.* 59:357–363.
7. Szent-Gyorgyi, A. G., V. N. Kalabokis, and C. L. Perreault-Micale. 1999. Regulation by molluscan myosins. *Mol. Cell. Biochem.* 190: 55–62.
8. Yamada, A., M. Yoshio, H. Kojima, and K. Oiwa. 2001. An in vitro assay reveals essential protein components for the ''catch'' state of invertebrate smooth muscle. *Proc. Natl. Acad. Sci. USA*. 98:6635–6640.
9. Tsutsui, Y., M. Yoshio, K. Oiwa, and A. Yamada. 2007. Striated muscle twitchin of bivalves has ''catchability'', the ability to bind thick filaments tightly to thin filaments, representing the catch state. *J. Mol. Biol.* 365:325–332.
10. Butler, T. M., S. U. Mooers, C. Li, S. Narayan, and M. J. Siegman. 1998. Regulation of catch muscle by twitchin phosphorylation: effects on force, ATPase, and shortening. *Biophys. J.* 75:1904–1914.
11. Butler, T. M., S. R. Narayan, S. U. Mooers, D. J. Hartshorne, and M. J. Siegman. 2001. The myosin cross-bridge cycle and its control by twitchin phosphorylation in catch muscle. *Biophys. J.* 80:415–426.
12. Lowy, J., and B. M. Millman. 1963. The contractile mechanism of the anterior byssus retractor of *Mytilus edulis*. *Proc. R. Soc. Lond. B Biol. Sci.* 246:105–148.
13. Galler, S., M. C. Hopflinger, O. Andruchov, O. Andruchova, and H. Grassberger. 2005. Effects of vanadate, phosphate and 2,3-butanedione monoxime (BDM) on skinned molluscan catch muscle. *Pflugers Arch.* 449:372–383.
14. Andruchova, O., M. C. Hopflinger, O. Andruchov, and S. Galler. 2005. No effect of twitchin phosphorylation on the rate of myosin head detachment in molluscan catch muscle: are myosin heads involved in the catch state? *Pflugers Arch.* 450:326–334.
15. Butler, T. M., S. U. Mooers, and M. J. Siegman. 2006. Catch force links and the low to high force transition of myosin. *Biophys. J.* 90: 3193–3202.
16. Shelud'ko, N. S., G. G. Matusovskaya, T. V. Permyakova, and O. S. Matusovsky. 2004. Twitchin, a thick-filament protein from molluscan catch muscle, interacts with F-actin in a phosphorylation-dependent way. *Arch. Biochem. Biophys.* 432:269–277.
17. Ruegg, J. C. 1971. Smooth muscle tone. *Physiol. Rev.* 51:201–248.
18. Vyas, T. B., S. U. Mooers, S. R. Narayan, J. C. Witherell, M. J. Siegman, and T. M. Butler. 1992. Cooperative activation of myosin by light chain phosphorylation in permeabilized smooth muscle. *Am. J. Physiol.* 263:C210–C219.
19. Vyas, T. B., S. U. Mooers, S. R. Narayan, M. J. Siegman, and T. M. Butler. 1994. Cross-bridge cycling at rest and during activation: turnover of myosin-bound ADP in permeabilized smooth muscle. *J. Biol. Chem.* 269:7316–7322.
20. Andruchov, O., O. Andruchova, and S. Galler. 2006. The catch state of mollusk catch muscle is established during activation: experiments on skinned fiber preparations of the anterior *byssus* retractor muscle of *Mytilus edulis L.* using the myosin inhibitors orthovanadate and blebbistatin. *J. Exp. Biol.* 209:4319–4328.
21. Walker, J. W., G. P. Reid, J. A. McCray, and D. R. Trentham. 1988. Photolabile 1-(nitrophenyl)ethyl phosphate esters of adenine nucleotide analogues. Synthesis and mechanism of photolysis. *J. Am. Chem. Soc.* 110:7170–7177.
22. Cheng, H. C., B. E. Kemp, R. B. Pearson, A. J. Smith, L. Misconi, S. M. Van Patten, and D. A. Walsh. 1986. A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *J. Biol. Chem.* 261:989–992.
23. Hai, C.-M., and R. A. Murphy. 1988. Cross-bridge phosphorylation and regulation of latch state in smooth muscle. *Am. J. Physiol.* 256: C99–C106.
24. Hopflinger, M. C., O. Andruchova, O. Andruchov, H. Grassberger, and S. Galler. 2006. Effect of pH on the rate of myosin head detachment in molluscan catch muscle: are myosin heads involved in the catch state? *J. Exp. Biol.* 209:668–676.
25. Baguet, F., and J. M. Gillis. 1968. Energy cost of tonic contraction in lamellibranch catch muscle. *J. Physiol.* 198:127–143.
26. Siegman, M. J., T. M. Butler, S. U. Mooers, and R. E. Davies. 1980. Chemical energetics of force development, force maintenance, and relaxation in mammalian smooth muscle. *J. Gen. Physiol.* 76:609–629.
27. Butler, T. M., M. J. Siegman, and S. U. Mooers. 1983. Chemical energy usage during shortening and work production in mammalian smooth muscle. *Am. J. Physiol.* 244:C234–C242.
28. Wingard, C. J., R. J. Paul, and R. A. Murphy. 1994. Dependence of ATP consumption on cross-bridge phosphorylation in swine carotid smooth muscle. *J. Physiol. (Lond.).* 481:111–117.
29. Aksoy, M. O., S. Mras, K. E. Kamm, and R. A. Murphy. 1983. Ca^{2+}, cAMP, and changes in myosin phosphorylation during contraction of smooth muscle. *Am. J. Physiol.* 245:C255–C270.
30. Fenn, W. O. 1923. A quantitative comparison between the energy liberated and the work performed by the isolated *sartorius* muscle of the frog. *J. Physiol.* 58:175–203.
31. Huxley, A. F. 1957. Muscle structure and theories of contraction. *Prog. Biophys. Biophys. Chem.* 7:255–318.

32. Nyitrai, M., and M. A. Geeves. 2004. Adenosine diphosphate and strain sensitivity in myosin motors. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 359:1867–1877.

33. Whittaker, M., E. M. Wilson-Kubalek, J. E. Smith, L. Faust, R. A. Milligan, and H. L. Sweeney. 1995. A 35-Å movement of smooth muscle myosin on ADP release. *Nature*. 378:748–751.

34. Jontes, J. D., E. M. Wilson-Kubalek, and R. A. Milligan. 1995. A 32 degree tail swing in brush border myosin I on ADP release. *Nature*. 378:751–753.

35. Smith, D. A., and M. A. Geeves. 1995. Strain-dependent cross-bridge cycle for muscle. *Biophys. J.* 69:524–537.

36. Geeves, M. A., C. Perreault-Micale, and L. M. Coluccio. 2000. Kinetic analyses of a truncated mammalian myosin I suggest a novel isomerization event preceding nucleotide binding. *J. Biol. Chem.* 275: 21624–21630.

37. Butler, T. M., S. R. Narayan, S. U. Mooers, and M. J. Siegman. 1995. Strain dependence of cross bridge kinetics in smooth muscle. *Biophys. J.* 68:A169.

38. Khromov, A. S., M. R. Webb, M. A. Ferenczi, D. R. Trentham, A. P. Somlyo, and A. V. Somlyo. 2004. Myosin regulatory light chain phosphorylation and strain modulate adenosine diphosphate release from smooth muscle myosin. *Biophys. J.* 86:2318–2328.

39. Fenn, W. O. 1924. The relation between the work performed and the energy liberated in muscular contraction. *J. Physiol.* 58:373–395.

40. Curtin, N. A., and R. E. Davies. 1975. Very high tension with very little ATP breakdown by active skeletal muscle. *J. Mechanochem. Cell Motil.* 3:147–154.

41. Butler, T. M., M. J. Siegman, and S. U. Mooers. 1984. Chemical energy usage during stimulation and stretch of mammalian smooth muscle. *Pflugers Arch.* 401:391–395.